FINAL REPORT

ON

PHOTOSYNTHETIC HALOPHILES FROM OWENS VALLEY

Contract NASw-1294

SGC 917FR-1

31 August 1966

Prepared for

CHIEF, ENVIRONMENTAL BIOLOGY (CODE SB)
Office of Space Science and Applications
National Aeronautics and Space Administration
Washington, D.C.

Prepared by

SPACE-GENERAL CORPORATION
9200 East Flair Drive
El Monte, California

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Approved by:

E. Mishuck
Senior Division Manager
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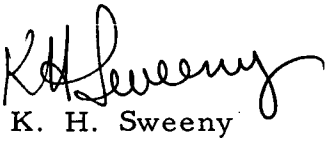

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ABSTRACT

Studies on Photosynthetic Halophiles
from Owens Lake

K. H. Sweeny

12255

Studies of growth of a halophilic, anaerobic, photosynthetic bacteria from an halite - thenordite - trona evaporite deposit in the Owens Valley of California have led to an increased understanding of the behavior of organisms under an extremely hostile environment. This small Chromatium was found to exist in small pockets in the crystal with dimensions approximately equal to the organism; the organism must therefore obtain water from the crystalline hydrate, or from water trapped in crystalline defects.

It has been noted that the growth cycle of the organism in moving from lag to log stage involves oxidation of sulfide in the brine, the formation of a swollen cell, and finally the reduction in size to the normal short rod. It may be hypothesized that water introduction into the cell is associated with the sulfur cycle. Bacteriochlorophyll production follows the sulfide oxidation and subsequent sulfur shower.

A remarkable tolerance to temperature extremes is shown by the Chromatium. Temperatures as high as 180°C have been resisted for short periods of time and 110°C or -35 to -55° storage appears to have little deleterious effect on growth. The reduction in pressure to about 30 mm. (a value reasonably approximating a Martian atmosphere) may actually accelerate growth, compared to an earth atmosphere.

These phenomenological studies strongly suggest the need for mechanistic investigations of the mode of water introduction under extreme environments, and of the mechanism of tolerance to extreme temperatures and pressures.

Author

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ILLUSTRATIONS

Section 1

INTRODUCTION

This report of progress in "Studies of Photosynthetic Halophiles from Owens Lake" is submitted in accordance with the requirements of Contract No. NASw 1294.

This study is aimed at an understanding of organism growth in an extreme environment; growth on Mars can be taken as an example of such extreme conditions. Two of the ways in which the Martian environment differs significantly from Earth are the scarcity of free water and the absence of atmospheric oxygen. This implies that Martian life must either occupy an occasional niche in which free water exists, or must concentrate water from sources in which, by terrestrial standards, it would be tightly bound and unavailable for life. On Mars, energy for synthesis and for the accumulation of water for synthesis would of necessity be derived from an anaerobic process, although, for thermodynamic reasons, being ultimately dependent upon the sun.

Studies of growth limitation by water or anaerobic photosynthesis under conditions in some way approaching Mars may be accomplished with a number of terrestrial organisms from a number of environments. However, only in rare cases can both be investigated simultaneously. The literature is replete with references on halophiles and on photosynthetic bacteria, but is notably lacking in publications on photosynthetic halophiles. A recent paper by Jannasch[1] and the report on the previous contract at Space-General on Chromatium from an Owens Valley evaporator pond[2] are exceptions. Other references include studies by Van Niel[3] and Baas-Becking[4] which report studies on halophilism within the genus Chromatium and Thiorhodaceae respectively from Searles Lake of the Owens River chain and from Owens Lake.

The characterization of growth behavior as related to water activity has been the specific objective of research discussed in this report. These studies have been carried out within the more general frame of reference of the program, which is concerned with the identification of mechanisms permitting the existence of both photosynthetic and sulfate reducing halophiles in the saline environment, and the extent and energetics of water limitation of growth of the photosynthetic species on salt crystals and in brines.

Three general classes of experiments are described in the sections to follow. In one series of experiments, the general characteristics of growth behavior are described. In a second series of experiments, an effort is being made to identify the precise environment which the microorganisms experience in the simulated Martian environment of the Owens Valley salt cake. The direct observation of organisms in their crystalline environment by a special microscopic technique has been one way of achieving this objective. The third class of experiments is concerned with establishing growth characteristics under a variety of hostile environmental conditions.

In the previous contract on this program, the purified isolate was identified as a member of a group of small Chromatium species promoting sulfur formation in the growth medium. The optimum water activity for rapid growth appeared to be 0. 95. It was further noted that in solutions of NaCl or sodium carbonates at pH 9. 5, growth appears to be a function of a_w while in sodium sulfate brines the response is related to a more complex property of the solute. The initial results of a theoretical study of halophilic growth indicated that, in the saturated solutions used, water does not move osmotically, but must be pumped into the cell. It was with this background that the current program on the relation between growth and activity of multiphasic brines and an investigation of the precise mechanism of water take-up by the cell was initiated.

As was indicated earlier, the study of the organisms from the Owens Lake brines offers the unique opportunity for study of photosynthetic halophiles, where growth limitation by water and anaerobic photosynthesis can be examined in a single system.

Section 2

MATERIALS AND METHODS

Methods used routinely during the investigations are described in the following section. Special procedures or specific deviations from these methods are described in the appropriate sections of the report.

The nutrient solutions for studies in solid and liquid media are defined in Table 1. Analytical grade sodium chloride, Na_2SO_4, $NaHCO_3$, Na_2CO, and commercial grade $NaHCO_3 \cdot Na_2CO_3 \cdot 2H_2O$ (trona) were added as required for experiments with artificial brines. Solid media for isolation and maintenance of isolates contained 60 grams NaCl, 20 grams $NaHCO_3 \cdot Na_2CO_3 \cdot 2H_2O$, 35 grams Na_2SO_4 and 15 grams agar per liter. Brines were sterilized by pasteurization for 1 minute at 85°C after filtration through 0.45 micron sterile Millipore filters. When solid salt phases were added, pasteurization was the only treatment used. The agar component of solid media was autoclaved separately and added to pasteurized brine at 45°C. In many of the experiments, synthetic brines having a water activity of 0.95 or 0.78 were used; the composition of these brines is given in Table 2.

Cultures were routinely incubated under 2450 fc illumination from 100 watt fluorescent lamps, which may not provide an optimal spectral distribution. Growth temperatures were $25 \pm 1°C$, or $32 \pm 1°C$.

Experiments with liquid media were carried out in 50 ml Erlenmeyer flasks containing 50 ml brine and provided with rubber stoppers. Pasteurization, nitrogen purging, and the H_2S reaction with oxygen were relied on to create conditions of anaerobiasis and low oxidation reduction potential. Nitrogen purging probably did not reduce oxygen tensions appreciably in the gaseous phase over the cultures. Enrichment cultures were carried through serial transfers in milk dilution bottles filled nearly to the rim, pasteurized, again filled to the rim with pasteurized medium, inoculated, and capped.

Table 1

COMPOSITION OF NUTRIENT SOLUTIONS

Component	Grams/Liter	
	Solid Medium	Brines
NH_4Cl	.4	1.0
K_2HPO_4 $3H_2O$	2.0	5.0
$MgSO_4$ $7H_2O$	.2	1.0
Na_2S $9H_2O$	4.8	2.4
$Na_2S_2O_3$	4.0	5.0
Fe	.001*	.001*
Minor Elements**		0.1 ml**

*As Fe citrate chelate
**Hutner's trace elements

Table 2

COMPOSITION OF SYNTHETIC BRINES

Substance	Composition, g/l	
	A_w 0.78	A_w 0.95
NaCl	285.0	35.20
Na_2SO_4	42.6	42.60
Na_2CO_3	9.54	9.54
Na HCO_3	15.96	15.96
NH_4Cl	1.0	1.0
$K_2HPO_4 \cdot 3H_2O$	5.0	5.0
Mg $SO_4 \cdot 7H_2O$	1.0	1.0
$N_aS \cdot 9H_2O$	2.4	2.4
$Na_2S_2O_3$	5.0	5.0
Minor Elements*	0.1 ml/l	0.1 ml/l

*Hutner's trace elements

Growth was evaluated by determination of optical density at 780 mμ and 525 mμ and by cell counts (Petroff-Hauser). Optical density data were taken by use of 1 cm round cells in a Bausch and Lomb Spectronic 340, for which a red sensitive photocell was provided. Values over 0.500 were obtained by dilution, optical density determination, and multiplication by the dilution factors. Generally, cultures were cut in a Waring blendor prior to analysis. This method has the disadvantage of being dependent on pigment synthesis, which may vary with cultural conditions. Oxidation reduction potentials and pH were determined with a pH meter and corrected for sodium low concentration when necessary.

Chemical analyses were performed according to methods in Reference 5 as follows: chloride by titration with mercuric nitrate; sulfide by methylene blue method, sulfate by the turbidimetric $BaSO_4$ procedure; and carbonate by electrometric determination of titration curves with .02N H_2SO_4. Potential interference by accompanying ions exerted an important influence over the choice of sample sizes and aliquots. Computations of molalities were dependent on measurement of specific gravity by weight or by hydrometer.

Section 3

RESULTS

In this section an attempt is made to summarize the data so as to give a consistent picture of the growth process of the studies aimed at establishing the precise environment which the organism experiences, and of the resistance of Chromatium to extreme conditions. The data upon which the summary is based is contained in the three quarterly reports on this program.

3.1 GROWTH PROCESS OF CHROMATIUM

The general growth characteristics of the halophilic Chromatium is summarized in Table 3. The characteristics of the growth curve are conventional, though an extended lag stage may be noted in some cases of extreme environment (discussed in Section 3.3). Photomicrographs illustrating the organisms during the phases of growth are given in Figure 1.

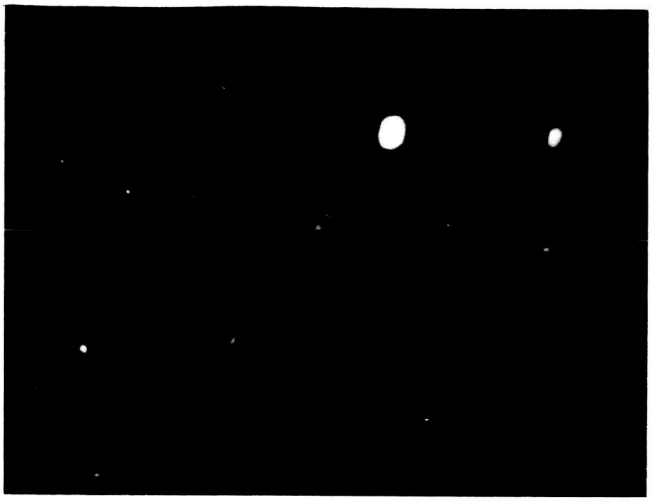
An important observation on this program has been that the disappearance of sulfide from solution leads to large glowing sulfur globules. This loss of sulfide in solution appears to proceed the transition from lag to log stages of growth.

It is observed that sulfur globules within cells can be correlated with growth and population density. During the first 30 days of growth there is a lag which is followed by a tremendous increase in growth accompanied by the utilization of sulfides present. At the time of the sulfur shower there is a pronounced increase of enlarged cells with sulfur globules followed by a decline with the onset of the pink color indicating the end of the extensive use of sulfides. After 35-40 days growth, cells lose their dependency upon the initial sulfur in the medium and the number of cells enlarged with sulfur globules are at a minimum. Only rarely will microscopic examination reveal cells glowing with sulfur globules into the stationary phase.

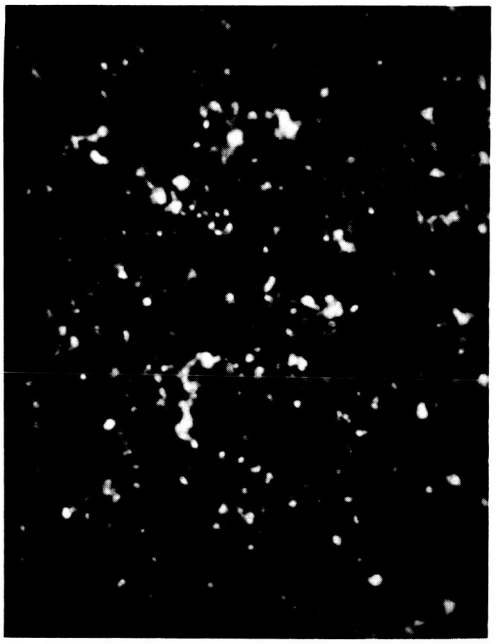
Table 3

CHARACTERISTICS OF CHROMATIUM CULTURES AT VARIOUS STAGES OF GROWTH

Stage of Growth	Cells/ml Populations	Cell-Appearance	Motility	Bacterio-Chlorophyll	Sulfide	pH
Lag	10^6 - 10^7	Cultures clear, cells short rod-shaped, ca. .6μ dia. x .8 -1.0μ long, little or no pink color.	Moderately active appear to move over complete micro-scope field	None	Starts to drop as growth starts	constant
Log	10^7 - 10^9	Cultures yellow, suspended sulfur particles in medium glowing sulfur globules in cells, cells range up to 10μ in dia., cells range in shape from spheroid to grape "cluster," color masked by flowing sulfur globules and in many cells linked end to end in groups of 2-3.	Sluggish, some rotate moderately rapidly. (ca 1/2 rps) Movement over restricted area	Move at start, bacterio-chlorophyll production starts at end of sulfur shower	Drops to 0 at beginning of log stage	Slight rise (.1 -.2 pH).
Stationary	10^9	Cultures pink to deep purple, suspended sulfur settles, cells long rods .6μ x 1 - 2μ long. Cells pink color	Very active and highly motile over large area.	high	0	constant
Decline	10^9 - 10^7	Cultures clear at top, cells settle to bottom of container. Cells long rods .6μ dia. x 1 - 1.5μ long. Cells pink color.	Movement less active, many non-motile cells	high	0	constant



LOG



STATIONARY

DECLINE

Figure 1. Photomicrographs of Chromatium at Different Stages of Growth (500X)

Chromatium in Aw 0.78 or Aw 0.95 brines over 60 days old do not appear to possess cells with glowing masses. The cells at this age revert back to normal size and shape. An analysis of the portion of cells containing the glowing sulfur globules as a function of the growth cycle is shown in Table 4.

A view of a cell containing the large mass of the sulfur globule is shown in Figure 2. In this view, the cell has swollen to a length of over 10 microns.

It is clear that the appearance of the sulfur globules is related to the growth cycle. A further observation, and one that is not easily quantitated, is that the size of the sulfur globules within a typical cell is also increasing. During the sulfur shower (9-21 days) the cell and sulfur globules may increase as much as 10-fold in diameter (from a nominal 1 μ to about 10 μ as is shown in Figure 2) and the microscopic appearance is that of a glowing yellow globule. Following the sulfur shower, the cells gradually revert to the nominal 1 μ size. During the shower, the cells take on various shapes and some are linked together, occasionally, resulting in chain-like configurations. At this time locomotion is very slow and sluggish, but the cells have shown minute peripheral activity. The sulfur shower and consequent loss of globules by the cell occurs within a time range of 24 to 72 hours. At this time the solution turns from yellow to cloudy pink and the lag phase of growth is observed. When this occurs the concentration of sulfides are reduced sharply. The individual cell becomes smaller in overall dimensions and movement can be described as a jerky back and forth pattern. These cells now resemble closely the cells found either in laboratory-grown or natually-occuring Owens Lake crystals.

Studies have been made in an effort to relate growth to the production of bacterio-chlorophyll. It was expected that this investigation would provide important information on the mechanism of energy transfer in the organism.

Table 4

SULFUR GLOBULES IN CHROMATIUM CULTURED
IN 0.78 and 0.95 A_w BRINES

Day	Cells x 10^6		% with Sulfur Globules		Color * of A_w 0.78 Cultures
	A_w 0.78	A_w 0.95	A_w 0.78	A_w 0.95	
0	3.0	4.0	2.0	2.0	Clear
2	21.0	22.0	5.0	5.0	Clear
5	17.6	21.0	14.	10.	Clear
9	13.0	17.8	35.	15.	Yellow
12	12.6	8.2	28.	20.	Yellow
14	21.4	10.8	32.	15.	Deep yellow
21	16.7	16.7	38.	15.	Deep yellow
26	17.5	14.7	36.	14.	Cloudy pink
29	220.	10.7	26.	13.	Cloudy pink
34	317.	9.7	20.	8.	Pink
41	605.	12.1	3.0	14.	Pink
43	650.	13.1	0.01	16.	Pink
48	460.	28.5	0	12.	Pink
54	410.	23.0	0	7.	Pink
61	--	--	0	0	Pink
64	--	--	0	0	Pink

*All 0.95 A_w cultures were clear.

Figure 2. Large Glowing Sulfur Globule Measuring over 10 Microns in Length from Chromatium Culture 15 Days Old

Data from analyses in aged cultures suggests that bacteriochlorophyll is associated with a pronounced increase in red-purple pigmentation. Observations and test results further show that the production of bacteriochlorophyll is evident after the sulfides have vanished; the two have been found together in appreciable amounts. It is possible that Chromatium are at first dependent to a degree upon sulfides for energy source and later upon the ability to synthesize bacteriochlorophyll.

The bacteriochlorophyll are determined spectrophotometrically from methanolic extracts[6]. Typical curves showing the presence of bacteriochlorophyll in later stages of growth is shown in Figure 3. The method of Van Niel appears satisfactory with the substitution of a modern spectrophotometer.

It is known that the addition of 25% Hcl to bacteriochlorophyll converts the material to bacteriophaephytin. Similar results have been obtained on prolonged exposure to fluorescent lamps (ca 3 days). This results is noted by a reduced absorbance at 760 and 600 mμ.

A need exists for a good solid medium in which the photosynthetic anaerobes can be made to grow. A new nutrient agar medium containing ethyl alcohol, malic acid, l-glutamic acid, yeast extract and reduced salt concentrations was examined for this purpose. The composition of the medium is given in Table 5. With this medium, growth was observed in 2 days with culture H-10 and in 3 days with culture H-10-1. These samples showed continued growth after over one month.

3.2 ESTABLISHMENT OF PRECISE ENVIRONMENT WHICH CHROMATIUM EXPERIENCES

It is believed that a significant step towards defining the mechanism of adaptation has been achieved with the first successful observation of the organism in its crystal environment.

The technique used is as follows: If a crystal is immersed in a liquid of the same refractive index, it becomes transparent. However, vacuoles, liquid, pockets, organisms, inclusions, or mixed salts having a

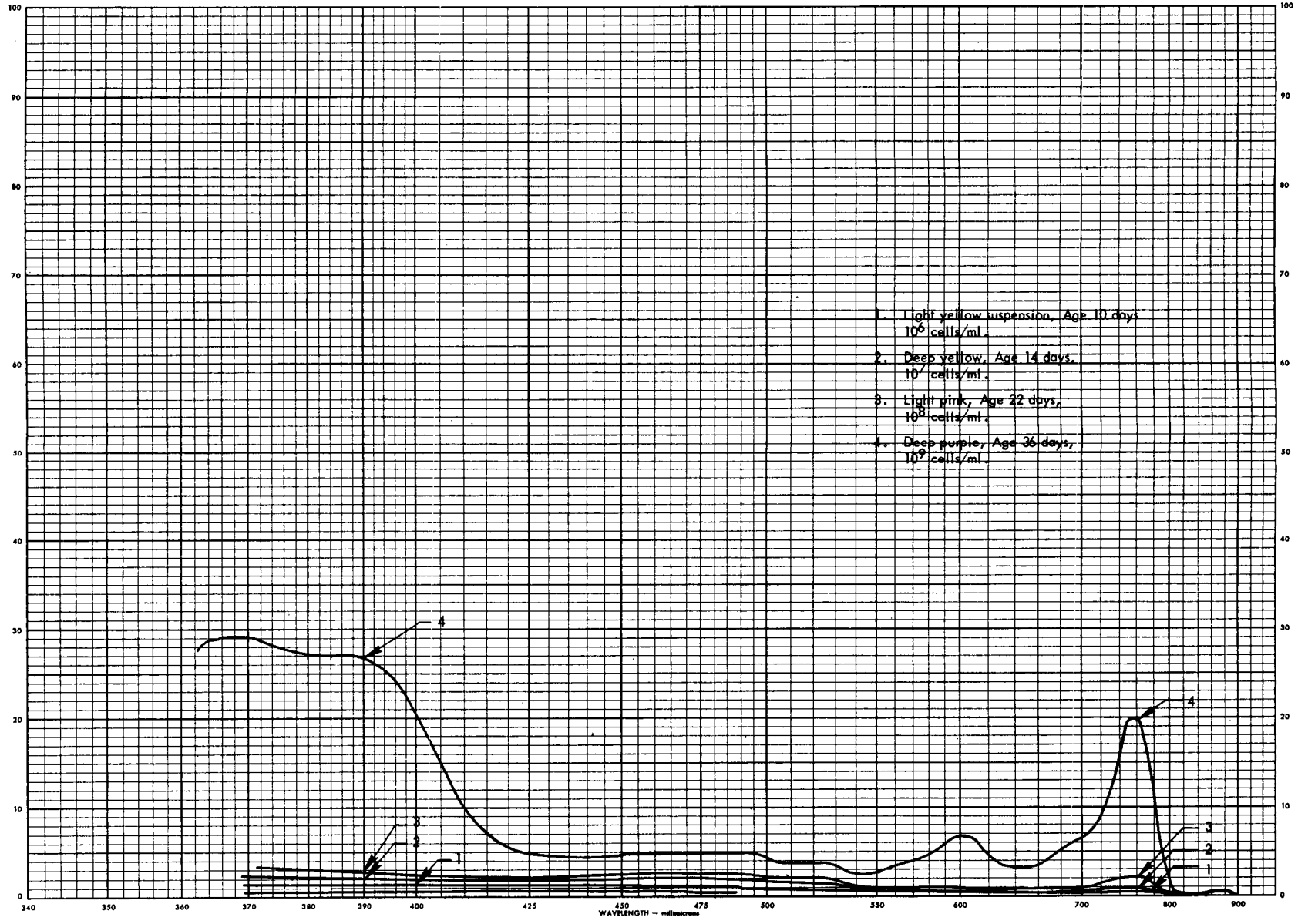


Figure 3. Spectrophotometric Trace for Bacteriochlorophyll Analysis at Various Stages of Growth

Table 5

COMPOSITION OF SOLID NUTRIENT
PHOTOSYNTHETIC MEDIUM

Substances	Concentration, g/l
Agar	18
Ethanol	5.0
Malic acid	1.0
l-Glutamic acid	0.5
Yeast extract	1,5
$NaHCO_3$	5.0
NaCl	3.0
$MgSO_4 \cdot 7H_2O$	1.0
$K_2HPO_4 \cdot 3H_2O$	5.0
Iron*	1.0 ml
Trace elements**	0.1 ml

* as iron citrate chelate

** Hutner's Trace elements

different refractive index will apparently "stand out", permitting easy observation under the microscope. In this study, isobutyl alcohol (η_D = 1.394) was used to "blank-out" mirabilite (η_D = 1.394, 1.396, 1.398) or Owens Valley salt cake. Cumulated experience from a number of observations is given in Table 6, while photomicrographs illustrative of behavior in Owens Lake Brine is shown in Figure 4 and in laboratory-recrystallized mirabilite in Figure 5. The motion of a Chromatium cell in a pocket in mirabilite is shown by the examination of Figure 6.

The examination of many samples has shown that Chromatium in Owens Lake crystals, either naturally occurring or recrystallized, are contained in pockets of essentially the same size as the organism. Hence no motion or brine can be observed. The environment then is that of the crystalline mass, and it can not be determined directly whether the water form is that of the water of hydration of the crystalline mass, or free water entrapped in defects in the crystal. It was pointed out in an analysis on the preceeding contract[2], that on a theoretical basis the preferred water form would be the oriented water of hydration.

A series of experiments were performed in an effort to establish experimentally the effect of free water versus equivalent water coordinated to the crystal as water of hydration. Experiments were performed near the transition temperatures of sodium sulfate, where the following reaction takes place.

$Na_2 SO_4 \cdot 10 H_2O \underset{\leftarrow}{\overset{32.384^{\circ}C}{\rightleftharpoons}} Na_2 SO_4 + 10 H_2O$ It was thought that experiments with mirabilite at 32° would involve coordinated water and similar tests at 33 · 5°C would present free water. However, these experiments have been inconclusive and a careful analysis of the phenomenon of the crystalline transition appears to suggest the reason for this. Although the observed behavior of free water and coordinated water appears at a precise temperature such that the sodium sulfate transition can be used as a temperature standard, the strength of the bonds holding the water to the salt have been weakening over a broad temperature range. It is probably necessary therefore to employ techniques such as the tagged atom approach outlined in Section 6 to establish the preferred route of entry of water into the organisms.

Table 6

CHARACTERISTICS OF ORGANISMS OBSERVED ENTRAPPED IN CRYSTALS

Type of Crystal	Organism Location in Crystal	Cell Appearance	Cell Movement	Pocket Appearance	Pocket Size
Naturally-occurring Owens Lake Cake	Uniformly spersed in pocket same size as organism	Oval, light pink to colorless, .6μ dia. x .8μ long	None	Shape and size of organism	.6μ dia. x .8μ long
Recrystallized in Owens Lake Brine	Uniformly dispersed in pockets same size as organism	Oval, colorless or faint pink, .6μ dia. x .8μ long	None	Shape and size of organism	.6μ dia. x .8μ long
Laboratory-grown Mirabilite	In pockets in crystal sometimes partly filled with brine. Some in layers in apparent crystal defects	Oval to short rods, pink color. .6μ dia. x 1-2μ long	Restricted Motion	Often half-filled with brine also cell debris seen	irregular - up to 10-20μ
Laboratory-grown from Synthetic brine	In small pockets	Oval, some short rods, pink color, .6μ dia. x 1μ long	Restricted Motion	Pockets so small could not tell if brine contained in it.	2-3μ

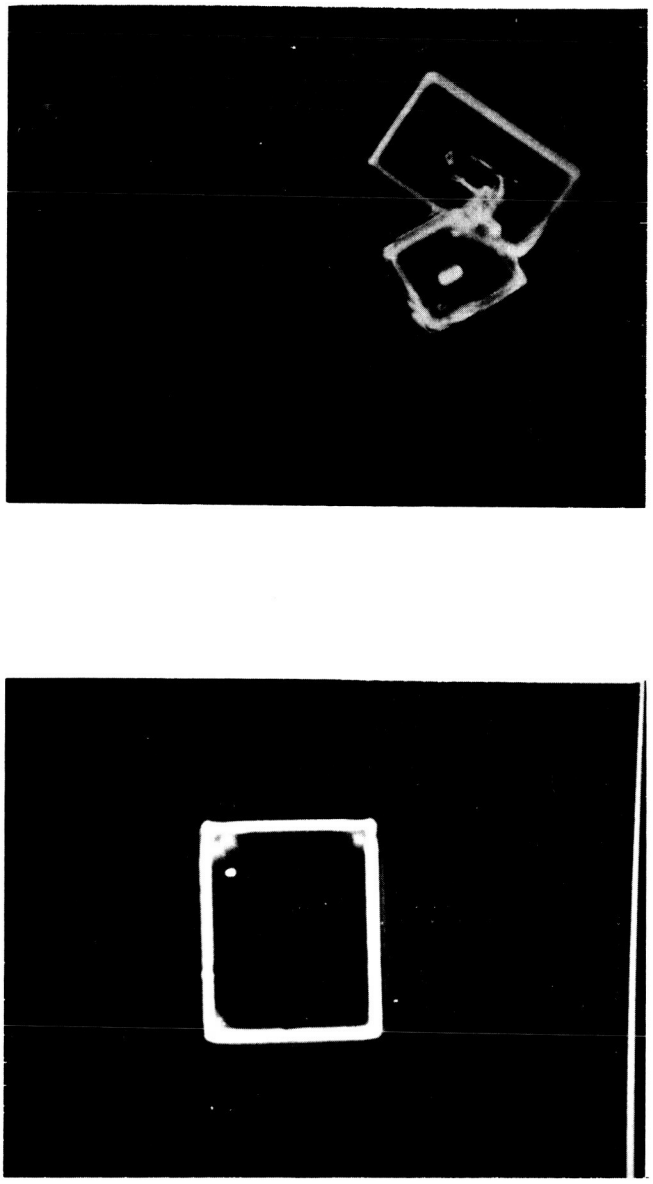
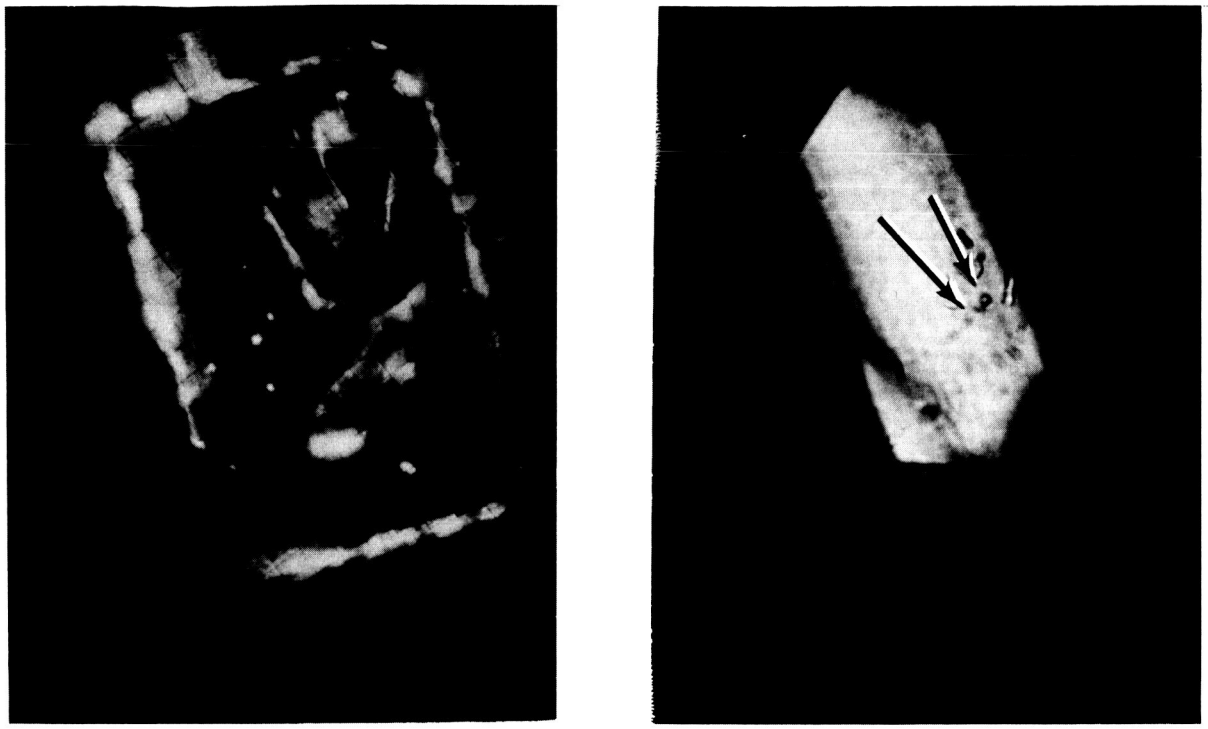


Figure 4. Photomicrograph Showing Organisms in Recrystallized Owens Lake Brine (500X)



a. Cells Glowing within Salt Crystal

b. Cell Trail

Figure 5. Photomicrographs of Chromatium Cells in Mirabilate Crystals

Figure 6. Photomicrographs Showing Movement of Chromatium within Cell Pocket (500X, Time Interval Between Photos, 95 Seconds)

3.3 RESISTANCE OF CHROMATIUM TO EXTREME ENVIRONMENT

Continued studies with the halophilie Chromatium has shown the organism to be remarkably resistant to a wide variety of extreme conditions. A summary of a number of environmental stress conditions is shown in Table 7.

In early experiments, organisms withstood dry oven temperatures of 180°C, apparently without damage while more recently, autoclaving for 15 min did not kill the organisms while longer autoclaving of 30 or 45 min resulted in death of the Chromatium. The organisms appear to survive deep freeze temperatures (-35 to -55°C for one month) satisfactorily, as well as exposure to 110°C environments.

An interesting result was obtained when samples were conditioned at a total pressure equal to the vapor pressure of synthetic brine (ca. 30 mm). The growth cycles were rapid and the extent of growth was at least equal to that at a terrestrial atmospheric pressure.

Although the organisms appear to withstand short exposure to dark, before transferring to the light, it was found that cultures kept in the dark for 25 days before transferring to the light did not survive.

A number of observations appear to indicate that the halophilic Chromatium is more resistant to extreme environment when it is entrapped in crystals, compared to behavior in brines or on solid media. It further appears that the oval or very short rod shaped cells are most resistant. When highly mobile rod-shaped cells were transferred from an A_w 0.95 brine to saturated Owens Lake Brine, for example, lysis of the cells was observed. The oval shaped cells appear to withstand the transfer, however.

Table 7

EFFECT ON ENVIRONMENTAL STRESS ON GROWTH OF HALOPHILIC CHROMATIUM

Type of Environment Stress	Growth Characteristics	Cell Shape	Cell Movement	Bacterio-chlorophyll Production
High Temperature				
110°C	normal growth, lag stage normal	rods	normal	normal
180°C	Extended lag and slow growth in log stage	initially short rods	normal	lower than normal
Autoclave, 15 psi	15 min - normal growth cycles	rods	normal	normal
steam	30-45 min - organ-isms killed	rods - broken distorted	none	none
Low Temperature				
-10 to -15°C	normal	rods	normal	normal
-55°C	Normal in crystals, little growth in frozen brine	short rods	normal	normal to slightly low
Reduced Pressure	Rapid/cycles	rods	more active than normal	normal
High Salt Concen-tration	Slow cycles - 16 day	short rods to oval	very sluggish	weak peaks
Light - dark	no growth, even when transferred to light	distorted and broken rods	none	none

Section 4

CONCLUSIONS

The adaptability of the halophilic photosynthetic chromatium from the concentrated brines and salt cakes of Owens Lake to a variety of extreme environments has been further demonstrated by experiments under this contract.

The growth characteristics of the organism involving the utilization of sulfide with the production of an enlarged sulfur globule followed by a reduction in size to form normal sized rods appears to be a key process in the transition from the lag to log stages of growth. Since the sulfur globules appear to be less dense than the normal organism, it may be suggested that the mode of water introduction into the cell may involve the transfer through a sulfur cycle.

The production of bacteriochlorophyll has also been studied and although the material is related to growth, bacteriochlorophyll does not appear until after the sulfides have vanished. Bacteriochlorophyll is associated with the red-purple pigmentation observed in later stages of growth. It is possible, therefore, that chromatium may be at first dependent to a degree upon sulfides as an energy source and later upon the ability to photosynthetically synthesize bacteriochlorophyll.

It is believed that a significant contribution to the understanding of the effect of the medium on growth was made when direct observations were made of the chromatium in their crystalline environment. The organisms in an Owens Lake environment, either as the salt cake or from recrystallized brine, are present in vacuoles in the crystal having dimensions approximately the same as the organism. Neither liquor in the pockets, nor motion of the cell could be observed. It appears that the environment which the organism experiences is that of the salt cake; it is not clear, however, whether this

includes water as a hydrate, or whether free water trapped in crystal defects is the preferred source. Organisms trapped in mirabilite crystals appear to be found in pockets with principal dimensions about ten times that of the organism. The pockets are typically half-filled with brine and the cells may move about these pockets.

Unusual resistance to a variety of environments is shown by the chromatium. Both high and low temperatures are withstood, and growth under pressures approximating Martian conditions may be greater than at an earth atmosphere. Light appears clearly necessary for growth.

Section 5

ANALYSIS OF RESULTS

This research appears significant to the practical NASA objectives as an example of work with life in an unusual, highly-stressed environment. Basic data has been obtained which establishes the unusual resistance of the photosynthetic halophilic chromatium to temperature extremes, reduced pressure, and minimal water.

Photomicrographic studies of the chromatium in a crystalline environment show clearly that the organisms in the Owens Lake salt cake are surviving without large quantities of water available, and it is suggested that water is consumed either from the hydrate salt crystal (probably the preferred form), or from free water in crystal defect sites. The phenomenological observations strongly suggest the necessity of conducting mechanistic studies of the mode of water assimilation by the organism, as well as the means by which the organism can withstand temperature and pressure extremes. Such studies should be valuable in establishing a basis for predicting life under extreme extraterrestrial conditions.

A further practical aspect of the program is that information is provided which should lead to optimal means for sampling and preserving samples from an extraterrestrial probe.

The efforts on this and the preceeding contract are leading toward a firm description of the limiting processes for life in terms of basic physicochemical processes. Efforts to date have shown the relation of water activity to solute composition, and a phase change has been suggested as a source of water. The successful completion of these and recommended future studies should lead to a firm understanding of the factors controlling life under extreme environments.

Section 6

RECOMMENDED FUTURE WORK

On the basis of the studies outlined in the preceeding sections, it appears that data have been obtained for which an understanding is required in order to permit the prediction of organism survival under extraterrestrial conditions. The strong need for mechanistic studies is evident and the recommended investigations are believed to provide a significant understanding of the behavior of organisms under extreme environmental conditions. Three tasks are briefly outlined. The first of these involves the use of tagged atoms to trace the movement of water into the organism. The relation between the water cycle and sulfur cycle would also be examined. The second task involves the precise establishment of the death curve of chromatium with simultaneous assay in an effort to determine the factors associated with survival. A structural comparison with less resistant organisms would also be made. The third task is associated with the binding forces that hold organisms to a soil substrate, the establishment of these forces, and a study of methods of freeing the organisms from a soil substrate.

6.1 MECHANISM OF WATER ABSORPTION BY HALOPHILIC CHROMATIUM

Important to the understanding of the means by which organisms can exist in a hostile environment is the manner in which they ingest water. It is important to establish the precise manner by which the water is taken up by the organism, since this provides a basis for predicting the conditions under which life is most likely to exist, and thus guides the experimenter along the most profitable lines in his exploration of hostile areas, such as Mars.

The work carried out under contract NASw 1294 has established that the sulfide content drops to zero at the point of the sulfur shower at the initiation of the log phase of growth and that the cells are observed to swell appreciably at this point with a decrease in density. It is also known that both the sulfide and sulfate ions carry coordinated water, a form suggested by an earlier

analysis as being the preferred form for water assimilation by the organism. It therefore seems plausible that the entrance of water into the organism may come from water held as water of hydration by the sulfur forms and that the transition sulfide-hydrate to sulfur may well represent the ingestion of water as well as sulfur oxidation.

It is therefore proposed to study the mode of water ingestion in the chromatium cells by following the path of tagged atoms. In this study, the sulfur can be followed by means of the S^{35} tag, and indeed tagged compounds suitable for use are readily available. The use of a tritium tag for the water seems most appropriate. The precise experiments would involve the utilization of the sulfide, and whether it is oxidized to sulfur or sulfate (or both), and the mode by which water passes into the cell. Either the direct counting of cells, the lysing of the cells prior to counting, or the extraction of particular components of the cell with subsequent radiological examination could be employed. The use by the organism of water that has been coordinated to the sulfide or sulfate, as well as free water in solution, would be examined similarly.

These experiments should lead to a firm evaluation of the need by the organisms for free water, and the ability of the organism to obtain water from chemically combined forms. Thus important conclusions can be drawn of the ability of organisms such as the chromatium species to survive under extreme conditions.

Similar experiments to those for sulfur can also be carried out with carbonates, in order that the effect of the water hydrated to the carbonate ion might be determined.

6.2 AN EXAMINATION OF THE FACTORS CONTROLLING SURVIVAL OF THE HALOPHILIC CHROMATIUM UNDER TEMPERATURE AND PRESSURE EXTREMES

An important finding of the studies under contract NASw 1294 has been the remarkable ability of the halophilic chromatium to survive under extremely hostile conditions. An investigation of the mode by which survival becomes limited may well offer important information relative to the ability of organisms to survive under extraterrestrial conditions.

It has been found under contract NASw 1294 that the chromatium has survived for days at 110°F and for brief periods as high as 180°F. Autoclaving for 15 min has not killed the organisms, while exposure to the autoclave for 30 min or longer resulted in loss of viability. Exposure to deep freeze temperatures or pressure reduced to about 30 mm has not resulted in inactivation of the microorganisms.

It is recommended, therefore, that a study be made of the growth characteristics as a function of temperature so that the precise temperature limits which the halophilic chromatium can withstand might be determined. Concurrent with this study, an examination of the cells will be made to determine the factors resulting in death. For example, an infra-red analysis of cell suspensions may well indicate such factors as dehydration or protein denaturation. An establishment of the composition of the resistant chromatium and the comparison with other common organisms would also be important.

The establishment of the growth characteristics under reduced pressure conditions would also be carried out, with a similar analysis to be made.

These studies should provide important leads to the extremes of temperature and pressure which microorganisms can withstand and therefore provide a basis for prediction of the possible existence of such materials in extraterrestrial sites. The mechanism of withstanding hostile environments would also be furthered by a study of this type.

6.3 AN INVESTIGATION OF SOIL-ORGANISM BINDING FORCES

An important consideration in obtaining samples of organisms from extraterrestrial sources is that the organism may well be bound to the soil and a quantity of this biologically inert medium may be expected to be found with any organism sample. Techniques are available, however, for establishing soil-organism binding forces and a method of freeing the organism from the soil can be suggested.

An earlier study by Pethica* has suggested a number of ways by which cells can adhere one to another. These include chemical bonds such as hydrogen, amide or ester bonds, ion-pair and ion-triplet formation, electrostatic attractions, van der Waal forces, etc. In all, twelve possible mechanisms are suggested for organism-to-organism attraction or repulsion, many of which are equally important as means for attaching organisms to soil particles. In addition, it can be seen that particles may adhere by the purely physical entrapment by which dissimilar particles in a matrix cannot be removed until the matrix is separated. A further physical phenomena could occur in selected cases in which a flagellate may physically attach to a soil particle. Earlier work by the investigators now at Space-General has shown that microscopic physical properties on particles as small as a few microns can be studied directly by developed but unpublished techniques.

It is proposed therefore to examine the binding forces between soil and selected microorganisms by means of direct measurements on a microscopic scale. The force between the soil and organism can be measured by means of a quartz-fiber balance (a single fiber in flexure), observing the behavior under the microscope. The paper by Pethica has suggested that calcium and magnesium bonds may play an important role, and that observations on small rod-like organisms have shown a preferred orientation which can well be attributed to electrostatic charge distributions. These factors would also be examined in the establishment of cell-soil particle forces.

With the establishment of the type and extent of forces binding the soil to organisms, means by which the organism can be freed from the soil can be examined. In the case of physical entrapment, a simple water leach may be sufficient to release the organisms while specific types of electrolytes may be employed to reduce surface charge attractions in other cases. Another means which should be examined is based on extensive work at Space-General under other contracts. This technique involves the partition between two

*Pethica, B. A., "The Physical Chemistry of Cell Adhesion," Experimental Cell Research, Suppl. 8, 123-140 (1961).

immiscible aqueous phases. The liquid partition technique has been found highly selective for separating bacteria from a variety of atmospheric background debris, including siliceous dusts.

This study should give definitive answers to the questions of the extent to which organisms might be bound to extraterrestrial soil and provide means for freeing the organisms from the soil debris for return to earth.

Section 7

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Section 8

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